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<b>(21) International Application Number:</b> PCT/US96/09001 <b>(22) International Filing Date:</b> 6 June 1996 (06.06.96)  <b>(30) Priority Data:</b> 08/465,968      6 June 1995 (06.06.95)      US  <b>(71) Applicant (for all designated States except US):</b> HUMAN GENOME SCIENCES, INC. [US/US]; 9410 Key West Avenue, Rockville, MD 20850 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ROSEN, Craig, A. [US/US]; 22400 Rolling Hill Road, Laytonsville, MD 20882 (US). HU, Jing-Shan [GB/US]; 16125 Howard Landing Drive, Gaithersburg, MD 20878 (US). CAO, Liang [GB/GB]; 18B Suncrest Tower, Monmouth Terrace, Hong Kong (HK).  <b>(74) Agents:</b> MULLINS, J., G.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US) et al.		<b>(81) Designated States:</b> AL, AU, BG, BR, BY, CA, CN, CZ, EE, FI, GE, IL, JP, KG, KP, KR, KZ, LT, LV, MD, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR 2		
<b>(57) Abstract</b>  Disclosed is a human VEGF2 polypeptide and DNA (RNA) encoding such VEGF2 polypeptides. Also provided is a procedure for producing such polypeptide by recombinant techniques and antibodies and antagonist against such polypeptide. Also disclosed is a method of using such polypeptide for stimulating wound healing and for vascular tissue repair. Also provided are methods of using the antagonists to inhibit tumor growth, inflammation and to treat diabetic retinopathy, rheumatoid arthritis and psoriasis. Diagnostic methods for detecting mutations in the VEGF2 coding sequence and alterations in the concentration of VEGF2 protein in a sample derived from a host are also disclosed.		

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### **Human Vascular Endothelial Growth Factor 2**

This application is a continuation-in-part of a previous application filed in the United States Patent and Trademark Office by Rosen, C. et al. on March 8, 1994 and assigned serial number 08/207,550.

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptide of the present invention has been identified as a member of the vascular endothelial growth factor family. More particularly, the polypeptide of the present invention is vascular endothelial growth factor 2, sometimes hereinafter referred to as "VEGF2." The invention also relates to inhibiting the action of such polypeptide.

The formation of new blood vessels, or angiogenesis, is essential for embryonic development, subsequent growth, and tissue repair. Angiogenesis, however, is an essential part of certain pathological conditions such as neoplasia, for example, tumors and gliomas, and abnormal angiogenesis is associated with other diseases such as inflammation,

rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, J. and Klagsbrun, M., Science 235:442-447, (1987)).

Both acidic and basic fibroblast growth factor molecules are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear (Folkman, J., 1993, Cancer Medicine pp. 153-170, Lea and Febiger Press). A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N., et al., Endocr. Rev. 13:19-32, (1992)), also known as vascular permeability factor (VPF). Vascular endothelial growth factor is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells.

The murine VEGF gene has been characterized and its expression pattern in embryogenesis has been analyzed. A persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, e.g., in choroid plexus and kidney glomeruli. The data was consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation (Breier, G. et al. Development, 114:521-532 (1992)).

VEGF is structurally related to the  $\alpha$  and  $\beta$  chains of platelet-derived growth factor (PDGF), a mitogen for mesenchymal cells and placenta growth factor (PLGF), an endothelial cell mitogen. These three proteins belong to the same family and share a conserved motif. Eight cysteine residues contributing to disulfide-bond formation are strictly conserved in these proteins. Alternatively spliced mRNAs have been identified for both VEGF, PLGF and PDGF and these different splicing products differ in biological activity and in receptor-binding specificity. VEGF and PDGF function as homo-dimers or hetero-dimers and bind to receptors which elicit intrinsic tyrosine kinase activity following receptor dimerization.

VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF206 are bound to heparin containing proteoglycans in the cell surface. The temporal and spatial expression of VEGF has been correlated with physiological proliferation of the blood vessels (Gajdusek, C.M., and Carbon, S.J., Cell Physiol., 139:570-579, (1989)); McNeil, P.L., Muthukrishnan, L., Warder, E., D'Amore, P.A., J. Cell. Biol., 109:811-822, (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L.B., et al., Clin. Invest. 89:244-253, (1989)). The factor can be isolated from pituitary cells and several tumor cell lines, and has been implicated in some human gliomas (Plate, K.H. Nature 359:845-848, (1992)). Interestingly, expression of VEGF121 or VEGF165 confers on Chinese hamster ovary cells the ability to form tumors in nude mice (Ferrara, N., et al., J. Clin. Invest. 91:160-170, (1993)). The inhibition of VEGF function by anti-VEGF monoclonal antibodies was shown to inhibit tumor growth in immune-deficient mice (Kim, K.J., Nature 362:841-844, (1993)). Further, a dominant-negative mutant of the VEGF receptor has been shown to inhibit growth of glioblastomas in mice.

Vascular permeability factor, has also been found to be responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF is an important factor in wound healing. Brown, L.F. et al., J. Exp. Med., 176:1375-9 (1992).

The expression of VEGF is high in vascularized tissues, (e.g., lung, heart, placenta and solid tumors) and correlates with angiogenesis both temporally and spatially. VEGF has also been shown to induce angiogenesis in vivo. Since angiogenesis is essential for the repair of normal tissues,

especially vascular tissues, VEGF has been proposed for use in promoting vascular tissue repair (e.g., in atherosclerosis).

U.S. Patent No. 5,073,492, issued December 17, 1991 to Chen et al., discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a hetero-dimer or homo-dimer. The protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European Patent Application No. 92302750.2, published September 30, 1992.

The polypeptides of the present invention have been putatively identified as a novel vascular endothelial growth factor based on amino acid sequence homology to human VEGF.

In accordance with one aspect of the present invention, there are provided novel mature polypeptides, as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof. The polypeptides of the present invention are of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the polypeptides of the present invention, including mRNAs, DNAs, cDNAs, genomic DNA as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with still another aspect of the present invention, there are provided processes for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of

the present invention, under conditions promoting expression of said proteins and subsequent recovery of said proteins.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide for therapeutic purposes, for example, to stimulate angiogenesis, wound-healing, and to promote vascular tissue repair.

In accordance with yet another aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, to inhibit the growth of tumors, to treat diabetic retinopathy, inflammation, rheumatoid arthritis and psoriasis.

In accordance with another aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to nucleic acid sequences of the present invention.

In accordance with another aspect of the present invention, there are provided methods of diagnosing diseases or a susceptibility to diseases related to mutations in nucleic acid sequences of the present invention and proteins encoded by such nucleic acid sequences.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Fig. 1 shows the cDNA sequence and the corresponding deduced amino acid sequence of the polypeptide of the present invention. The standard one letter abbreviations for amino acids are used. Sequencing was performed using 373 Automated DNA Sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97%.

Fig. 2 is an illustration of the amino acid sequence homology between the polypeptide of the present invention and other members of the human PDGF/VEGF family. The boxed areas indicate the conserved sequences and the location of the eight conserved cysteine residues.

Fig. 3 shows a photograph of a gel after in vitro transcription, translation and electrophoresis of the polypeptide of the present invention. Lane 1: <sup>14</sup>C and rainbow M.W. marker; Lane 2: FGF control; Lane 3: VEGF2 produced by M13-reverse and forward primers; Lane 4: VEGF2 produced by M13 reverse and VEGF-F4 primers; Lane 5: VEGF2 produced by M13 reverse and VEGF-F5 primers.

Fig. 4. VEGF2 polypeptide is expressed in a baculovirus system consisting of Sf9 cells. Protein from the medium and cytoplasm of cells were analyzed by SDS-PAGE under reducing and non-reducing conditions.

Fig. 5. The medium from Sf9 cells infected with a nucleic acid sequence of the present invention was precipitated and the resuspended precipitate was analyzed by SDS-PAGE and was stained with coomassie brilliant blue.

Fig. 6. VEGF2 was purified from the medium supernatant and analyzed by SDS-PAGE in the presence or absence of the reducing agent  $\beta$ -mercaptoethanol and stained by coomassie brilliant blue.

Fig. 7. Reverse phase HPLC analysis of purified VEGF2 using a RP-300 column (0.21 x 3 cm, Applied Biosystems,



Inc.). The column was equilibrated with 0.1% trifluoroacetic acid (Solvent A) and the proteins eluted with a 7.5 min gradient from 0 to 60% Solvent B, composed of acetonitrile containing 0.07% TFA. The protein elution was monitored by absorbance at 215 nm (Red line) and 280 nm (Blue line). The percentage of Solvent B is shown by Green line.

Fig. 8 illustrates the effect of partially-purified VEGF2 protein on the growth of vascular endothelial cells in comparison to basic fibroblast growth factor.

Fig. 9 illustrates the effect of purified VEGF2 protein on the growth of vascular endothelial cells.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer), as well as intervening sequences (introns) between individual coding segments (exons).

In accordance with one aspect of the present invention, there are provided isolated nucleic acid molecules (polynucleotides) which encode for the mature polypeptides having the deduced amino acid sequence of Figure 1 or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 97161 on May 24, 1995 or for polypeptides which have fewer amino acid residues than those showing in Figure 1.

A polynucleotide encoding a polypeptide of the present invention may be obtained from early stage human embryo (week 8 to 9) osteoclastomas, adult heart or several breast cancer cell lines. The polynucleotide of this invention was discovered in a cDNA library derived from early stage human embryo week 9. It is structurally related to the VEGF/PDGF family. VEGF2 contains an open reading frame encoding a protein of 419 amino acid residues of which approximately the first 23 amino acid residues are the putative leader sequence such that the mature protein comprises 396 amino acids, and which protein exhibits the highest amino acid sequence

homology to human vascular endothelial growth factor (30% identity), followed by PDGF $\alpha$  (23%) and PDGF $\beta$  (22%).

It is particularly important that all eight cysteines are conserved within all four members of the family (see boxed areas of Figure 2). In addition, the signature for the PDGF/VEGF family, PXCXXXXRCXGCCN, (SEQ ID NO:3) is conserved in VEGF2 (see Figure 2).

The VEGF2 polypeptide of the present invention is meant to include the full length polypeptide and polynucleotide sequence which encodes for any leader sequences and for active fragments of the full length polypeptide. Active fragments are meant to include any portions of the full length amino acid sequence which have less than the full 419 amino acids of the full length amino acid sequence as shown in SEQ ID No. 2 and Figure 2, but still contain the eight cysteine residues shown conserved in Figure 2 and such fragments still contain VEGF2 activity.

There are at least two alternatively spliced VEGF2 mRNA sequences present in normal tissues. The size of the two VEGF2 mRNA sequences which correspond to the full-length and truncated version respectively are shown in Figure 3, lane 5 shows two bands indicating the presence of the alternatively spliced mRNA encoding the VEGF2 polypeptide of the present invention.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition

of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of

Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a polypeptides which have the deduced amino acid sequence of Figure 1 or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 or that encoded by the deposited cDNA, means a polypeptide which retains the conserved motif of VEGF proteins as shown in Figure 2 and essentially the same biological function or activity.

The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides or synthetic polypeptides, preferably recombinant polypeptides.

The fragment, derivative or analog of the polypeptide of Figure 1 or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature

polypeptide or (v) one in which comprises fewer amino acid residues shown in SEQ ID No. 2 and retains the conserved motif and yet still retains activity characteristic of the VEGF family of polypeptides. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid

sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the VEGF2 genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and



phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli, lac or trp, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma;

adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pBluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNBO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a

mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and

a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GRM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone"

sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and

lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

As shown in Figures 8 and 9, the VEGF2 polypeptide of SEQ ID No. 2, minus the initial 46 amino acids, is a potent mitogen for vascular endothelial cells and stimulates their growth and proliferation. The results of a Northern blot analysis performed for the VEGF2 nucleic acid sequence encoding this polypeptide wherein 20  $\mu$ g of RNA from several human tissues were probed with  $^{32}$ P-VEGF2, illustrates that this protein is actively expressed in the heart and lung which is further evidence of mitogenic activity.

Accordingly, VEGF2 may be employed to promote angiogenesis, for example, to stimulate the growth of transplanted tissue where coronary bypass surgery is performed. VEGF2 may also be employed to promote wound healing, particularly to re-vascularize damaged tissues or stimulate collateral blood flow during ischemia and where new capillary angiogenesis is desired. VEGF2 may be employed to treat full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, VEGF2 may be employed to treat full-thickness burns and injuries where a skin graft or flap is used to repair such burns and injuries. VEGF2 may also be employed for use

in plastic surgery, for example, for the repair of lacerations from trauma and cuts in association with surgery.

Along these same lines, VEGF2 may be employed to induce the growth of damaged bone, periodontium or ligament tissue. VEGF2 may also be employed for regenerating supporting tissues of the teeth, including cementum and periodontal ligament, that have been damaged by disease and trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, VEGF2 may be employed in association with surgery and following the repair of cuts. It may also be employed for the treatment of abdominal wounds where there is a high risk of infection.

VEGF2 may be employed for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, VEGF2 can be applied to the surface of the graft or at the junction to promote the growth of vascular endothelial cells. VEGF2 may also be employed to repair damage of myocardial tissue as a result of myocardial infarction. VEGF2 may also be employed to repair the cardiac vascular system after ischemia. VEGF2 may also be employed to treat damaged vascular tissue as a result of coronary artery disease and peripheral and CNS vascular disease.

VEGF2 may also be employed to coat artificial prostheses or natural organs which are to be transplanted in the body to minimize rejection of the transplanted material and to stimulate vascularization of the transplanted materials.

VEGF2 may also be employed for vascular tissue repair, for example, that occurring during arteriosclerosis and required following balloon angioplasty where vascular tissues are damaged.

VEGF2 nucleic acid sequences and VEGF2 polypeptides may also be employed for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, and for the production of diagnostics and therapeutics to

treat human disease. For example, VEGF2 may be employed for *in vitro* culturing of vascular endothelial cells, where it is added to the conditional medium in a concentration from 10 pg/ml to 10 ng/ml.

Fragments of the full length VEGF2 gene may be used as a hybridization probe for a cDNA library to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Probes of this type generally have at least 50 base pairs, although they may have a greater number of bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete VEGF2 gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the VEGF2 gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

This invention provides methods for identification of VEGF2 receptors. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to VEGF2, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to VEGF2. Transfected cells which are grown on glass slides are exposed to labeled VEGF2. VEGF2 can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis.



Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, labeled VEGF2 can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing VEGF2 is then excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

This invention is also related to a method of screening compounds to identify those which are VEGF2 agonists or antagonists. An example of such a method takes advantage of the ability of VEGF2 to significantly stimulate the proliferation of human endothelial cells in the presence of the comitogen Con A. Endothelial cells are obtained and cultured in 96-well flat-bottomed culture plates (Costar, Cambridge, MA) in a reaction mixture supplemented with Con-A (Calbiochem, La Jolla, CA). Con-A, polypeptides of the present invention and the compound to be screened are added. After incubation at 37°C, cultures are pulsed with 1  $\mu$ Ci of  $^3$ [H]thymidine (5 Ci/mmol; 1 Ci = 37 BGq; NEN) for a sufficient time to incorporate the  $^3$ [H] and harvested onto glass fiber filters (Cambridge Technology, Watertown, MA). Mean  $^3$ [H]-thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant  $^3$ [H]thymidine incorporation, as compared to a control assay where the compound is excluded, indicates stimulation of endothelial cell proliferation.

To assay for antagonists, the assay described above is performed and the ability of the compound to inhibit <sup>3</sup>[H]thymidine incorporation in the presence of VEGF2 indicates that the compound is an antagonist to VEGF2. Alternatively, VEGF2 antagonists may be detected by combining VEGF2 and a potential antagonist with membrane-bound VEGF2 receptors or recombinant receptors under appropriate conditions for a competitive inhibition assay. VEGF2 can be labeled, such as by radioactivity, such that the number of VEGF2 molecules bound to the receptor can determine the effectiveness of the potential antagonist.

Alternatively, the response of a known second messenger system following interaction of VEGF2 and receptor would be measured and compared in the presence or absence of the compound. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis. In another method, a mammalian cell or membrane preparation expressing the VEGF2 receptor is incubated with labeled VEGF2 in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured.

Potential VEGF2 antagonists include an antibody, or in some cases, an oligonucleotide, which bind to the polypeptide and effectively eliminate VEGF2 function. Alternatively, a potential antagonist may be a closely related protein which binds to VEGF2 receptors, however, they are inactive forms of the polypeptide and thereby prevent the action of VEGF2. Examples of these antagonists include a negative dominant mutant of the VEGF2 polypeptide, for example, one chain of the hetero-dimeric form of VEGF2 may be dominant and may be mutated such that biological activity is not retained. An example of a negative dominant mutant includes truncated versions of a dimeric VEGF2 which is capable of interacting with another dimer to form wild type VEGF2, however, the

resulting homo-dimer is inactive and fails to exhibit characteristic VEGF activity.

Another potential VEGF2 antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of VEGF2. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the VEGF2 polypeptide (Antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of VEGF2.

Potential VEGF2 antagonists also include small molecules which bind to and occupy the active site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonists may be employed to treat limit angiogenesis necessary for solid tumor metastasis.

The mRNA encoding for VEGF2 is found to be expressed at moderate levels in at least two breast tumor cell lines which

is indicative of the role of VEGF2 polypeptides in the malignant phenotype. Gliomas are also a type of neoplasia which may be treated with the antagonists of the present invention.

The antagonists may also be used to treat chronic inflammation caused by increased vascular permeability. In addition to these disorders, the antagonists may also be employed to treat retinopathy associated with diabetes, rheumatoid arthritis and psoriasis.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The VEGF2 polypeptides and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide or agonist or antagonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical

compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions are administered in an amount of at least about 10  $\mu\text{g/kg}$  body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10  $\mu\text{g/kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The VEGF2 polypeptides, and agonists or antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptide *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells such as bone marrow cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide *ex vivo*, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding the polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo*, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding a polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and  $\beta$ -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the  $\beta$ -actin promoter; and human growth hormone

promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317,  $\psi$ -2,  $\psi$ -AM, PA12, T19-14X, VT-19-17-H2,  $\psi$ CRE,  $\psi$ CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO<sub>4</sub> precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

This invention is also related to the use of the VEGF2 gene as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in VEGF2 nucleic acid sequences.

Individuals carrying mutations in the VEGF2 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and

autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding VEGF2 can be used to identify and analyze VEGF2 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled VEGF2 RNA or alternatively, radiolabeled VEGF2 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers et al., Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton et al., PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.



In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of VEGF2 protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease, for example, abnormal cellular differentiation. Assays used to detect levels of VEGF2 protein in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan, et al., Current Protocols in Immunology, 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the VEGF2 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein, such as, bovine serum albumen. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any VEGF2 proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to VEGF2. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of VEGF2

protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to VEGF2 are attached to a solid support. Polypeptides of the present invention are then labeled, for example, by radioactivity, and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of VEGF2 in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay VEGF2 is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the VEGF2. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphism's) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those

hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., *Human Chromosomes: a Manual of Basic Techniques*. Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man* (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptide corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies binding the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the

manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described by the method of Graham, F. and Van der Eb, A., *Virology*, 52:456-457 (1973).

#### Example 1

##### Expression pattern of VEGF2 in human tissues and breast cancer cell lines

Northern blot analysis was carried out to examine the levels of expression of the VEGF2 gene in human tissues and human breast cancer cell lines. Total cellular RNA samples were isolated with RNazol™ B system (Biotech Laboratories, Inc.). About 10 µg of total RNA isolated from each breast

tissue and cell line specified was separated on 1% agarose gel and blotted onto a nylon filter, (Molecular Cloning, Sambrook Fritsch, and Maniatis, Cold Spring Harbor Press, 1989). The labeling reaction was done according to the Stratagene Cloning Systems, Inc., Prime-It kit with 50 ng DNA fragment. The labeled DNA was purified with a Select-G-50 column from 5 Prime -- 3 Prime, Inc, Boulder, CO, USA. The filter was then hybridized with radioactively labeled full length VEGF2 gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub> and 7 % SDS overnight at 65°C. After washing twice at room temperature and twice at 60°C with 0.5 X SSC, 0.1 % SDS, the filters were then exposed at -70°C overnight with an intensifying screen. A message of 1.6 Kd was observed in 2 breast cancer cell lines.

#### Example 2

##### Cloning and expression of VEGF2 using the baculovirus expression system

The DNA sequence encoding the VEGF2 protein without 46 amino acids at the N-terminus, see ATCC #97161, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence TGT AAT ACG ACT CAC TAT AGG GAT CCC GCC ATG GAG GCC ACG GCT TAT GC (SEQ ID NO:4) and contains a BamH1 restriction enzyme site (in bold) and 17 nucleotide sequence complementary to the 5' sequence of VEGF2 (nt. 150-166).

The 3' primer has the sequence GATC TCT AGA TTA GCT CAT TTG TGG TCT (SEQ ID NO:5) and contains the cleavage site for the restriction enzyme XbaI and 18 nucleotides complementary to the 3' sequence of VEGF2, including the stop codon and 15 nt sequence before stop codon.

The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101, Inc., La Jolla, CA). The fragment was then digested with the

endonuclease BamHI and XbaI and then purified again on a 1% agarose gel. This fragment was ligated to pAcGP67A baculovirus transfer vector (PHarming) at the BamHI and XbaI sites. Through this ligation, VEGF2 cDNA was cloned in frame with the signal sequence of baculovirus gp67 gene and was located at the 3' end of the signal sequence in the vector. This is designated pAcGP67A-VEGF2.

To clone VEGF2 with the signal sequence of gp67 gene to the PRG1 vector for expression, VEGF2 with the signal sequence and some upstream sequence were excised from the pAcGP67A-VEGF2 plasmid at the Xho restriction endonuclease site located upstream of the VEGF2 cDNA and at the XbaI restriction endonuclease site by XhoI and XbaI restriction enzyme. This fragment was separated from the rest of vector on a 1% agarose gel and was purified using "Geneclean" kit. It was designated F2.

The PRG1 vector (modification of pVL941 vector) is used for the expression of the VEGF2 protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI, SmaI, XbaI, BglII and Asp718. A site for restriction endonuclease XhoI is located upstream of BamHI site. The sequence between XhoI and BamHI is the same as that in pAcGp67A (static on tape) vector. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from E.coli is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by



viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid was digested with the restriction enzymes XboI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. E.coli HB101 cells were then transformed and bacteria identified that contained the plasmid (pBac gp67-VEGF2) with the VEGF2 gene using the enzymes BamHI and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5  $\mu$ g of the plasmid pBac gp67-VEGF2 was cotransfected with 1.0  $\mu$ g of a commercially available linearized baculovirus ("BaculoGold" baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1  $\mu$ g of BaculoGold<sup>™</sup> virus DNA and 5  $\mu$ g of the plasmid pBac gp67-VEGF2 were mixed in a sterile well of a microtiter plate containing 50  $\mu$ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10  $\mu$ l Lipofectin plus 90  $\mu$ l Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum

was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the virus was added to the cells, blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200  $\mu$ l of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-gp67-VEGF2 at a multiplicity of infection (MOI) of 1. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5  $\mu$ Ci of  $^{35}$ S-methionine and 5  $\mu$ Ci  $^{35}$ S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Protein from the medium and cytoplasm of the Sf9 cells was analyzed by SDS-PAGE under reducing and non-reducing conditions. See Figure 4. The medium was dialyzed against 50 mM MES, pH 5.8. Precipitates were obtained after dialysis and resuspended in 100 mM NaCitrate, pH 5.0. The resuspended

precipitate was analyzed again by SDS-PAGE and was stained with Coomassie Brilliant Blue. See Figure 5.

The medium supernatant was also diluted 1:10 in 50 mM MES, pH 5.8 and applied to an SP-650M column (1.0 x 6.6 cm, Toyopearl) at a flow rate of 1 ml/min. Protein was eluted with step gradients at 200, 300 and 500 mM NaCl. The VEGF2 was obtained using the elution at 500 mM. The eluate was analyzed by SDS-PAGE in the presence or absence of reducing agent,  $\beta$ -mercaptoethanol and stained by Coomassie Brilliant Blue. See Figure 6.

### Example 3

#### Expression of Recombinant VEGF2 in COS cells

The expression of plasmid, VEGF2-HA is derived from a vector pCDNA1/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron and polyadenylation site. A DNA fragment encoding the entire VEGF2 precursor and a HA tag fused in frame to its 3' end was cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A. Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37:767, (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding VEGF2, ATCC # 97161, was constructed by PCR using two primers: the 5' primer (CGC GGA TCC ATG ACT GTA CTC TAC CCA) (SEQ ID NO:6) contains a BamHI site followed by 18 nucleotides of VEGF2 coding sequence starting from the initiation codon; the 3' sequence (CGC TCT

AGA TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA CTC GAG GCT CAT  
TTG TGG TCT 3') (SEQ ID NO:7) contains complementary  
sequences to an XbaI site, HA tag, XhoI site, and the last 15  
nucleotides of the VEGF2 coding sequence (not including the  
stop codon). Therefore, the PCR product contains a BamHI  
site, coding sequence followed by an XhoI restriction  
endonuclease site and HA tag fused in frame, a translation  
termination stop codon next to the HA tag, and an XbaI site.  
The PCR amplified DNA fragment and the vector, pCDNAI/Amp,  
were digested with BamHI and XbaI restriction enzyme and  
ligated. The ligation mixture was transformed into E. coli  
strain SURE (Stratagene Cloning Systems, La Jolla, CA 92037)  
the transformed culture was plated on ampicillin media plates  
and resistant colonies were selected. Plasmid DNA was  
isolated from transformants and examined by restriction  
analysis for the presence of the correct fragment. For  
expression of the recombinant VEGF2, COS cells were  
transfected with the expression vector by DEAE-DEXTRAN method  
(J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A  
Laboratory Manual, Cold Spring Laboratory Press, (1989)).  
The expression of the VEGF2-HA protein was detected by  
radiolabelling and immunoprecipitation method (E. Harlow, D.  
Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor  
Laboratory Press, (1988)). Cells were labelled for 8 hours  
with <sup>35</sup>S-cysteine two days post transfection. Culture media  
was then collected and cells were lysed with detergent (RIPA  
buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC,  
50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984))).  
Both cell lysate and culture media were precipitated with an  
HA specific monoclonal antibody. Proteins precipitated were  
analyzed on 15% SDS-PAGE gels.

#### Example 4

The effect of partially-purified VEGF2 protein on the growth  
of vascular endothelial cells

On day 1, human umbilical vein endothelial cells (HUVEC) were seeded at  $2-5 \times 10^4$  cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium was replaced with M199 containing 10% FBS, 8 units/ml heparin. VEGF2 protein of SEQ ID NO. 2 minus the initial 45 amino acid residues, (VEGF) and basic FGF (bFGF) were added, at the concentration shown. On days 4 & 6, the medium was replaced. On day 8, cell number was determined with a Coulter Counter (See Figure 8).

#### Example 5

##### The effect of purified VEGF2 protein on the growth of vascular endothelial cells

On day 1, human umbilical vein endothelial cells (HUVEC) were seeded at  $2-5 \times 10^4$  cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium was replaced with M199 containing 10% FBS, 8 units/ml heparin. Purified VEGF2 protein of SEQ ID No. 2 minus initial 45 amino acid residues was added to the medium at this point. On days 4 & 6, the medium was replaced with fresh medium and supplements. On day 8, cell number was determined with a Coulter Counter (See Figure 9).

#### Example 6

##### Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room

temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

PMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: HU, ET AL.
- (ii) TITLE OF INVENTION: Human Vascular Endothelial Growth Factor 2
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN, CECCHI, STEWART & OLSTEIN
  - (B) STREET: 6 BECKER FARM ROAD
  - (C) CITY: ROSELAND
  - (D) STATE: NEW JERSEY
  - (E) COUNTRY: USA
  - (F) ZIP: 07068
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: 3.5 INCH DISKETTE
  - (B) COMPUTER: IBM PS/2
  - (C) OPERATING SYSTEM: MS-DOS
  - (D) SOFTWARE: WORD PERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/465,968
  - (B) FILING DATE: 6 JUN 95
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/207,550
  - (B) FILING DATE: 8 MAR 1994



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(C) REFERENCE/DOCKET NUMBER: 325800-288

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- (A) TELEPHONE: 201-994-1700  
(B) TELEFAX: 201-994-1744

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 1674 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCTTCCAC CATGCACTCG CTGGGCTTCT TCTCTGTGGC GTGTTCTCTG CTCGCCGCTG 60  
CGCTGCTCCC GGGTCCTCGC GAGGCGCCCG CCGCCGCCGC CGCCTTCGAG TCCGGACTCG 120  
ACCTCTCGGA CGCGGAGCCC GACGCGGGCG AGGCCACGGC TTATGCAAGC AAAGATCTGG 180  
AGGAGCAGTT ACGGTCTGTG TCCAGTGTAG ATGAAGTCAT GACTGTACTC TACCCAGAAT 240  
ATTGGAATAAT GTACAAGTGT CAGCTAAGGA AAGGAGGCTG GCAACATAAC AGAGAACAGG 300  
CCAACCTCAA CTCAAGGACA GAAGAGACTA TAAATTTTCG TGCAGCACAT TATAATACAG 360  
AGATCTTGAA AAGTATTGAT AATGAGTGGA GAAAGACTCA ATGCATGCCA CGGGAGGTGT 420  
GTATAGATGT GGGGAAGGAG TTTGGAGTCG CGACAAACAC CTTCTTTAAA CCTCCATGTG 480  
TGTCCTCTCA CAGATGTAGG GGTGCTGCA ATAGTGAGGG GCTGCAGTGC ATGAACACCA 540  
GCACGAGCTA CCTCAGCAAG ACGTTATTTG AAATTACAGT GCCTCTCTCT CAAGGCCCCA 600  
AACCAGTAAC AATCAGTTTT GCCAATCACA CTTCTGCGG ATGCATGTCT AAAGTGGATG 660  
TTTACAGACA AGTTCATTCC ATTATTAGAC GTTCCCTGCC AGCAACACTA CCACAGTGTC 720  
AGGCAGCGAA CAAGACCTGC CCCACCAATT ACATGTGGAA TAATCACATC TGCAGATGCC 780  
TGGCTCAGGA AGATTTTATG TTTTCCTCGG ATGCTGGAGA TGAATCAACA GATGGATTCC 840  
ATGACATCTG TGGACCAAAC AAGGAGCTGG ATGAAGAGAC CTGTCAGTGT GTCTGCAGAG 900  
CGGGGCTTCG GCCTGCCAGC TGTGGACCCC ACAAAGAACT AGACAGAAAC TCATGCCAGT 960  
GTGCTGTAA AAACAACTC TCCCCAGCC AATGRGGGGC CAACCGACAA TTTGATGAAA 1020

ACACATGCCA GTGTGTATGT AAAAGAACCT GCCCCAGAAA TCAACCCCTA AATCCTGGAA 1080  
 AATGTGCCTG TGAATGTACA GAAAGTCCAC AGAAATGCTT GTTAAAAGGA AAGAAGTTCC 1140  
 ACCACCAAAC ATGCAGCTGT TACAGACGGC CATGTACGAA CCGCCAGAAG GCTTGTGAGC 1200  
 CAGGATTTTC ATATAGTGAA GAAGTGTGTC GTTGTGTCCC TTCATATTGG CAAAGACCAC 1260  
 AATGAGCTA AGATTGTACT GTTTTCCAGT TCATCGATTT TCTATTATGG AAAACTGTGT 1320  
 TGCCACAGTA GAACTGTCTG TGAACAGAGA GACCCITGTG GGTCCATGCT AACAAAGACA 1380  
 AAAGTCTGTC TTTCTGAAC CATGTGGATA ACTTTACAGA AATGGACTGG AGCTCATCTG 1440  
 CAAAAGGCCT CTTGTAAAGA CTGGTTTTCT GCCAATGACC AAACAGCCAA GATTTTCCTC 1500  
 TTGTGATTTT TTTAAAAGAA TGACTATATA ATTTATTTCC ACTAAAAATA TTGTTTCTGC 1560  
 ATTCATTTTT ATAGCAACAA CAATTGGTAA AACTCACTGT GATCAATATT TTTATATCAT 1620  
 GCAAAATATG TTTAAAATAA AATGAAAATT GTATTATAAA AAAAAAAAAA AAAA 1674

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 419 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS:

(D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: PROTEIN

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met His Ser Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala  
 -45 -40 -35  
 Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe  
 -30 -25 -20 -15  
 Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala  
 -10 -5 1  
 Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser  
 5 10 15  
 Ser Val Asp Glu Leu Met Thr Val Lys Tyr Pro Glu Tyr Trp Lys Met  
 20 25 30  
 Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln  
 35 40 45 50  
 Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala  
 55 60 65  
 His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys  
 70 75 80

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Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe
      85                      90                      95
Gly Val Ala Thr Asn Thr Phe Lys Pro Pro Cys Val Ser Val Tyr
      100                    105                    110
Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr
      115                    120                    125                    130
Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu
      135                    140                    145
Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser
      150                    155                    160
Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile
      165                    170                    175
Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn
      180                    185                    190
Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys
      195                    200                    205                    210
Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser
      215                    220                    225
Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu
      230                    235                    240
Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys
      245                    250                    255
Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys
      260                    265                    270
Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu
      275                    280                    285                    290
Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro
      295                    300                    305-
Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys
      310                    315                    320
Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr
      325                    330                    335
Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser
      340                    345                    350
Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Gln Arg Pro
      355                    360                    365                    370
Gln Met Ser

```

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 14 AMINO ACIDS

(B) TYPE: AMINO ACID

- (C) STRANDEDNESS:
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Xaa Cys Val Xaa Xaa Xaa Arg Cys Xaa Gly Cys Cys Asn

5

10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 50 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTAATACGA CTCACTATAG GGATCCCGCC ATGGAGGCCA CGGCTTATGC 50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 28 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCTCTAGA TTAGCTCATT TGTGGTCT 28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 27 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCGGATCCA TGACTGTACT CTACCCA

27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 60 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAC TCGAGGCTCA TTTGTGGTCT 60

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 196 AMINO ACIDS  
(B) TYPE: AMINO ACID  
(C) STRANDEDNESS:  
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Arg Thr Leu Ala Cys Leu Leu Leu Leu Gly Cys Gly Tyr Leu

5

10

15

Ala His Val Leu Ala Glu Glu Ala Glu Ile Pro Arg Glu Val Ile

20	25	30
Glu Arg Leu Ala Arg Ser Gln Ile His	Ser Ile Arg Asp Leu Gln	
35	40	45
Arg Leu Leu Glu Ile Asp Ser Val Gly	Ser Glu Asp Ser Leu Asp	
50	55	60
Thr Ser Leu Arg Ala His Gly Val His	Ala Thr Lys His Val Pro	
65	70	75
Glu Lys Arg Pro Leu Pro Ile Arg Arg	Lys Arg Ser Ile Glu Glu	
80	85	90
Ala Val Pro Ala Val Cys Lys Thr Arg	Thr Val Ile Tyr Glu Ile	
95	100	105
Pro Arg Ser Gln Val Asp Pro Thr Ser	Ala Asn Phe Leu Ile Trp	
110	115	120
Pro Pro Cys Val Glu Val Lys Arg Cys	Thr Gly Cys Cys Asn Thr	
125	130	135
Ser Ser Val Lys Cys Gln Pro Ser Arg	Val His His Arg Ser Val	
140	145	150
Lys Val Ala Lys Val Glu Tyr Val Arg	Lys Lys Pro Lys Leu Lys	
155	160	165
Glu Val Gln Val Arg Leu Glu Glu His	Leu Glu Cys Ala Cys Ala	
170	175	180
Thr Thr Ser Leu Asn Pro Asp Tyr Arg	Glu Glu Asp Thr Asp Val	
185	190	195
Arg		

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 241 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS:

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Asn Arg Cys Trp Ala Leu Phe Leu Ser Leu Cys Cys Tyr Leu

5	10	15
Arg Leu Val Ser Ala Glu Gly Asp Pro Ile Pro Glu Glu Leu Tyr		
20	25	30
Glu Met Leu Ser Asp His Ser Ile Arg Ser Phe Asp Asp Leu Gln		
35	40	45
Arg Leu Leu His Gly Asp Pro Gly Glu Glu Asp Gly Ala Glu Leu		
50	55	60
Asp Leu Asn Met Thr Arg Ser His Ser Gly Gly Glu Leu Glu Ser		
65	70	75
Leu Ala Arg Gly Arg Arg Ser Leu Gly Ser Leu Thr Ile Ala Glu		
80	85	90
Pro Ala Met Ile Ala Glu Cys Lys Thr Arg Thr Glu Val Phe Glu		
95	100	105
Ile Ser Arg Arg Leu Ile Asp Arg Thr Asn Ala Asn Phe Leu Val		
110	115	120
Trp Pro Pro Cys Val Glu Val Gln Arg Cys Ser Gly Cys Cys Asn		
125	130	135
Asn Arg Asn Val Gln Cys Arg Pro Thr Gln Val Gln Leu Arg Pro		
140	145	150
Val Gln Val Arg Lys Ile Glu Ile Val Arg Lys Lys Pro Ile Phe		
155	160	165
Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu Ala Cys Lys Cys		
170	175	180
Glu Thr Val Ala Ala Ala Arg Pro Val Thr Arg Ser Pro Gly Gly		
185	190	195
Ser Gln Glu Gln Arg Ala Lys Thr Pro Gln Thr Arg Val Thr Ile		
200	205	210
Arg Thr Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg Lys		
215	220	225
Phe Lys His Thr His Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly		
230	235	240

Ala

(2) INFORMATION FOR SEQ ID NO:10:

(1) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 231 AMINO ACIDS  
 (B) TYPE: AMINO ACID  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met	Asn	Phe	Leu	Leu	Ser	Trp	Val	His	Trp	Ser	Leu	Ala	Leu	Leu	5	10	15
Leu	Tyr	Leu	His	His	Ala	Lys	Trp	Ser	Gln	Ala	Ala	Pro	Met	Ala	20	25	30
Glu	Gly	Gly	Gly	Gln	Asn	His	Glu	Val	Val	Lys	Phe	Met	Asp	Val	35	40	45
Tyr	Gln	Arg	Ser	Tyr	Cys	His	Pro	Ile	Glu	Thr	Leu	Val	Asp	Ile	50	55	60
Phe	Gln	Glu	Tyr	Pro	Asp	Glu	Ile	Glu	Tyr	Ile	Phe	Lys	Pro	Ser	65	70	75
Cys	Val	Pro	Leu	Met	Arg	Cys	Gly	Gly	Cys	Cys	Asn	Asp	Glu	Gly	80	85	90
Leu	Glu	Cys	Val	Pro	Thr	Glu	Glu	Ser	Asn	Ile	Thr	Met	Gln	Ile	95	100	105
Met	Arg	Ile	Lys	Pro	His	Gln	Gly	Gln	His	Ile	Gly	Glu	Met	Ser	110	115	120
Phe	Leu	Gln	His	Asn	Lys	Cys	Glu	Cys	Arg	Pro	Lys	Lys	Asp	Arg	125	130	135
Ala	Arg	Gln	Glu	Lys	Lys	Ser	Val	Arg	Gly	Lys	Gly	Lys	Gly	Gln	140	145	150
Lys	Arg	Lys	Arg	Lys	Lys	Ser	Arg	Tyr	Lys	Ser	Trp	Ser	Val	Tyr	155	160	165
Val	Gly	Ala	Arg	Cys	Cys	Leu	Met	Pro	Trp	Ser	Leu	Pro	Gly	Pro	170	175	180
His	Pro	Cys	Gly	Pro	Cys	Ser	Glu	Arg	Arg	Lys	His	Leu	Phe	Val	185	190	195
Gln	Asp	Pro	Gln	Thr	Cys	Lys	Cys	Ser	Cys	Lys	Asn	Thr	Asp	Ser	200	205	210



[illegible]

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:2;
  - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
  - (c) a polynucleotide fragment of the polynucleotide of (a) or (b).
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 2 which encodes the polypeptide as set forth in SEQ ID NO:2.
4. The polynucleotide of Claim 2 which encodes the polypeptide comprising -46 to 373 as set forth in SEQ ID NO:2.
5. The polynucleotide of Claim 2 which encodes the polypeptide comprising 1 to 373 as set forth in SEQ ID NO:2.
6. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide which encodes a mature polypeptide encoded by the DNA contained in ATCC Deposit No. 97161;
  - (b) a polynucleotide which encodes a polypeptide expressed by the DNA contained in ATCC Deposit No. 97161;
  - (c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and
  - (d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).

7. A vector containing the DNA of Claim 2.
8. A host cell genetically engineered with the vector of Claim 7.
9. A process for producing a polypeptide comprising: expressing from the host cell of Claim 8 the polypeptide encoded by said DNA.
10. A process for producing cells capable of expressing a polypeptide comprising transforming or transfecting the cells with the vector of Claim 7.
11. A polypeptide selected from the group consisting of (i) a polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof; (ii) a polypeptide comprising amino acid 1 to amino acid 373 of SEQ ID NO:2; and (iii) a polypeptide encoded by the cDNA of ATCC Deposit No. 97161 and fragments, analogs and derivatives of said polypeptide.
12. A compound effective as an agonist for the polypeptide of claim 11.
13. A compound effective as an antagonist against the polypeptide of claim 11.
14. A method for the treatment of a patient having need of VEGF2 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 11.
15. The method of Claim 14 wherein said therapeutically effective amount of the polypeptide is administered by

providing to the patient DNA encoding said polypeptide and expressing said polypeptide in vivo.

16. A method for the treatment of a patient having need of VEGF2 comprising: administering to the patient a therapeutically effective amount of the compound of claim 12.

17. A method for the treatment of a patient having need to inhibit VEGF2 comprising: administering to the patient a therapeutically effective amount of the antagonist of Claim 13.

18. A process for diagnosing a disease or a susceptibility to a disease related to expression of the polypeptide of claim 11 comprising:

determining a mutation in the nucleic acid sequence encoding said polypeptide.

19. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 11 in a sample derived from a host.

20. A method for identifying compounds which bind to and activate or inhibit a receptor for the polypeptide of claim 11 comprising:

contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with a compound to be screened under conditions to permit binding to the receptor; and

determining whether the compound binds to and activates or inhibits the receptor by detecting the presence or absence of a signal generated from the interaction of the compound with the receptor.

FIGURE 1A

FIGURE 1B

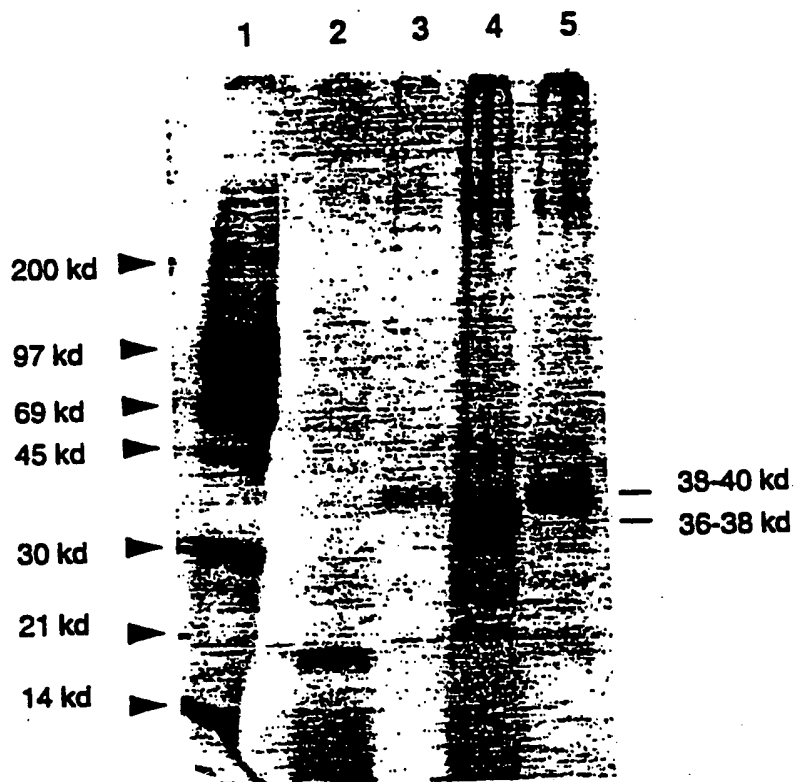
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CAAAAGGCCTCTTGTAAGACTGGTTTTCTGCCAATGACCAAACAGCCAAGATTTTCCTC  
1441 -----+----- 1500  
GTTTTCCGGAGAACATTTCTGACCAAAGACGGTTACTGGTTTGTCTGGTTCTAAAAGGAG  
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1501 -----+----- 1560  
AACACTAAAGAAATTTTCTTACTGATATATTAAATAAGGTGATTTTATAACAAAGACG  
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1561 -----+----- 1620  
TAAGTAAAAATATCGTTGTTGTTAACCATTTTGAGTGACACTAGTTATAAAAAATATAGTA  
GCAAAATATGTTTAAAATAAAATGAAAATTGTATTTATAAAAAAAAAAAAAAAAAA  
1621 -----+----- 1674  
CGTTTTATACAAATTTTATTTTACTTTTAACATAAATATTTTTTTTTTTTTTTT

FIGURE 1C

	1				50
Pdgfa	.MRTLACILL	LCCGYLAHVL	AEEAEIPREV	IERLARSOIE	SIRDLQRLLE
Pdgfb	MNRCA.LFL	SLCCYLRLVS	AEGDPIPEEL	YEMLSDESR	SFDOLQRLLE
Vegf	.....MNFLL	SWVHSLAEL	LY.....	.....	.LHAKWSQA
Vegf2	.....MTV	LYPEYWMYK	CQ.....	.....	.LRKGGWQHN
	51				100
Pdgfa	IDSVGSEDSL	DTSLRAGVE	ATREVPKRP	LPTRKRRI.	.....ZEAVP
Pdgfb	GDP.GEEDGA	ELDLNMTRE	SGGELES...	.LARGRRSLG	SLTIAEPAMI
Vegf	APMAE.....	.....GGGQ	NHREVVKPM	.VYQR.....	.....
Vegf2	REQANLNSRT	EETIKFAAAH	INTEILKSID	NEWRK.....	.....
	101				150
Pdgfa	AVKTRTVLY	EIPRSQVDPT	SANFLIWPFC	VEVKRCTGCC	ETSSVKQPS
Pdgfb	AEKTRTEVF	EISRLIDRT	MANFLVWPPC	VEVQRCSGCC	NHNVQCRPT
Vegf	SYHPITLV	DIFQEYFDEI	..EYIFNPSC	VPLNRCSGCC	NOEGLECVPT
Vegf2	TQMPREVC	DVGKEFGVAT	..NTFFNPFC	VSVYRCGGCC	NSGLQCHNT
	151				200
Pdgfa	RVEHRSVKA	KVEYVRKPK	LKEVQVRLE	HLKCA.....	AT.....
Pdgfb	QVQLRFPQVR	KIEIVRKPY	PKKATVTLED	HLAKC.....	ETVAAARPT
Vegf	EESNITHQIM	RIK.PH..QG	QHIGEMSLQ	HNKCEPRPK	DRARQEKSV
Vegf2	STSYLSKTLF	EIT.VPLSQG	PKPVTISFAN	ETSCRMKSL	DVYRQVESII
	201				250
Pdgfa	....TSLNPD	YREEDTDVR.	.....	.....	.....
Pdgfb	RSPGGSQQR	AKTPQTRVTI	RTVVRVRPPK	GKRRKPKTE	DKTALKETLG
Vegf	RK.....	.GKGQKPKK	KSRYKSWSVY	VGARCCLPW	SLPGPH...
Vegf2	RRSLPATLPQ	CQAANKTCPT	HYMWNHICR	CLAQEDFMPS	SDAGDDSTDG
	251				300
Pdgfa	.....	.....	.....	.....	.....
Pdgfb	A.....	.....	.....	.....	.....
Vegf	....CGP...	.....	.....CSE	RRKHLFVQDP	QTCRCCKMT
Vegf2	FEDICGPNKE	LDEETCQVC	RAGLRPASC	PKEL....DR	NSCQCVCNK
	301				350
Pdgfa	.....	.....	.....	.....	.....
Pdgfb	.....	.....	.....	.....	.....
Vegf	..DSRCARQ	LELNERTCRC	DKPRR.....	.....	.....
Vegf2	LFPSQCGANR	.EFDENTCQC	VCRCTCPNQ	PLNPGKACE	CTESPQKCLL
	351				398
Pdgfa	.....	.....	.....	.....	.....
Pdgfb	.....	.....	.....	.....	.....
Vegf	.....	.....	.....	.....	.....
Vegf2	KGKRFHQTC	SCYRRPCTNR	QKACEPGFSY	SEEVCRVPS	YWQRPQMS

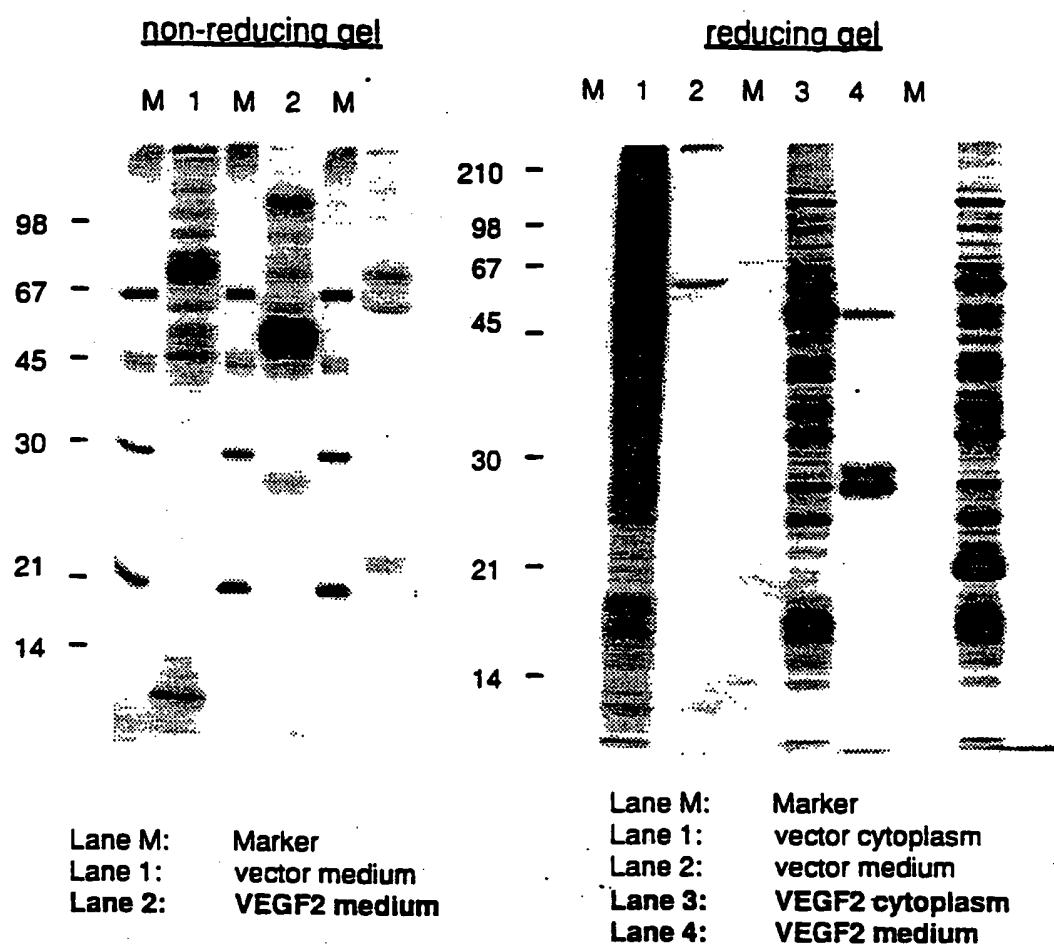
FIGURE 2





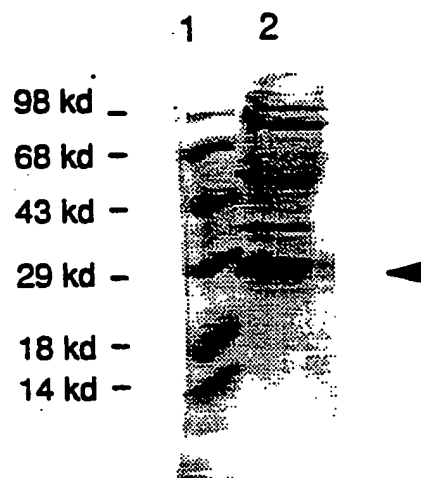
Lane 1: 14-C and rainbow M.W. marker  
Lane 2: FGF control  
Lane 3: VEGF2 (M13-reverse & forward primers)  
Lane 4: VEGF2 (M13-reverse & VEGF-F4 primers)  
Lane 5: VEGF2 (M13-reverse & VEGF-F5 primers)

FIGURE 3

**Figure****Expression of VEGF2 in a baculovirus system.****FIGURE 4**

**Figure**

**Analysis of crude VEGF2 protein from the conditioned medium by SDS-PAGE.**



Lane 1: Molecular weight marker  
Lane 2: Precipitates containing VEGF2.

FIGURE 5

**Figure**

**Analysis and purification of VEGF2 (HG401-2b) protein from conditioned medium.**

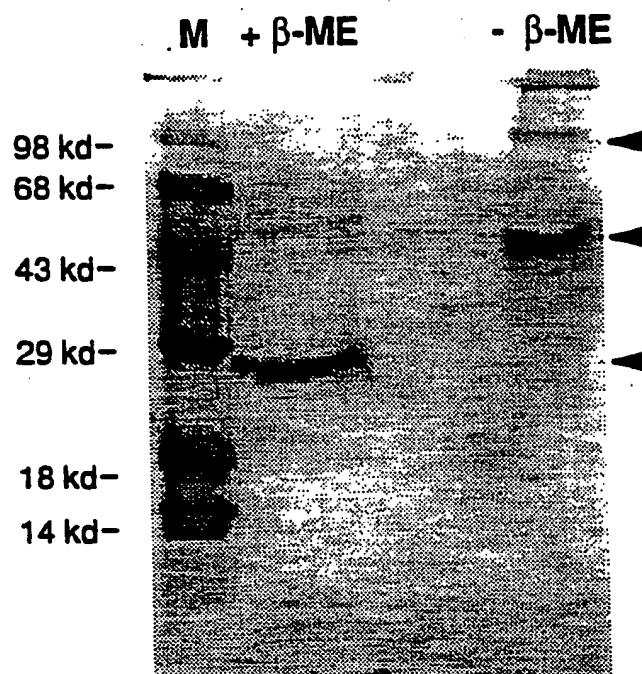


FIGURE 6

## Figure

Reverse phase HPLC analysis of purified VEGF2 (HG401-2B).

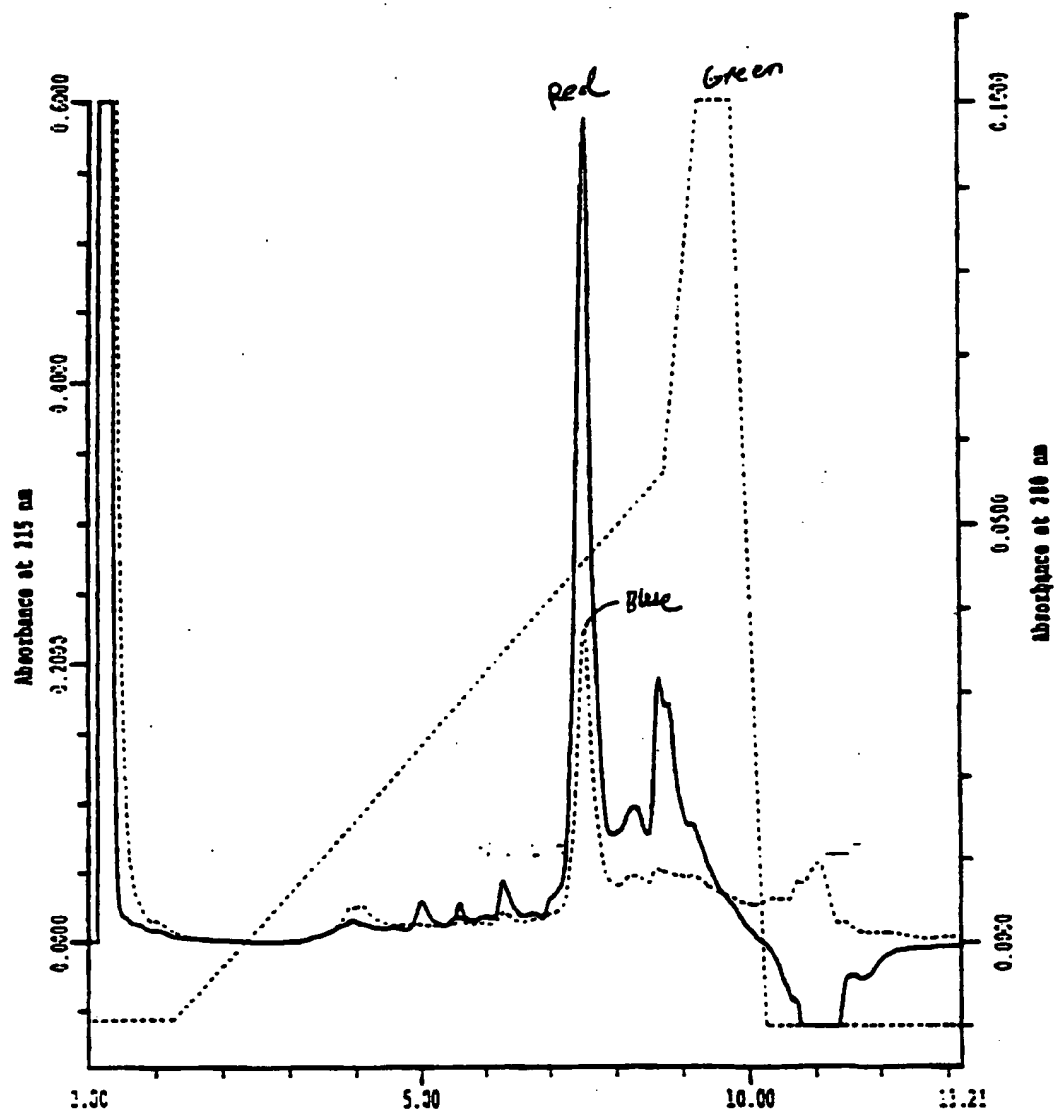


FIGURE 7

The effect of partially purified VEGF2 protein on the growth of vascular endothelial cells.

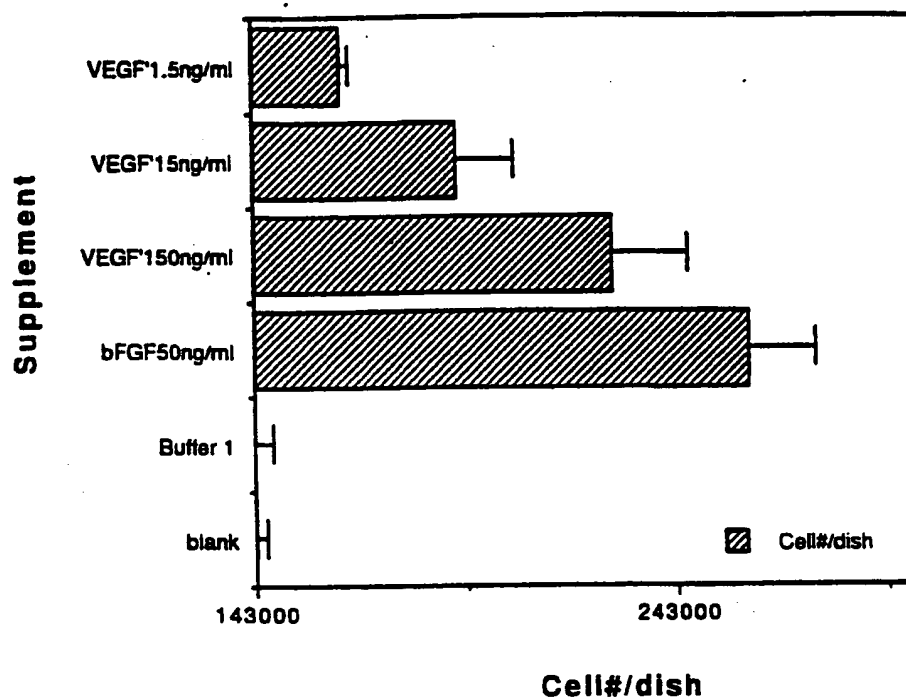


FIGURE 8

The effect of purified VEGF2 protein on the growth of vascular endothelial cells.

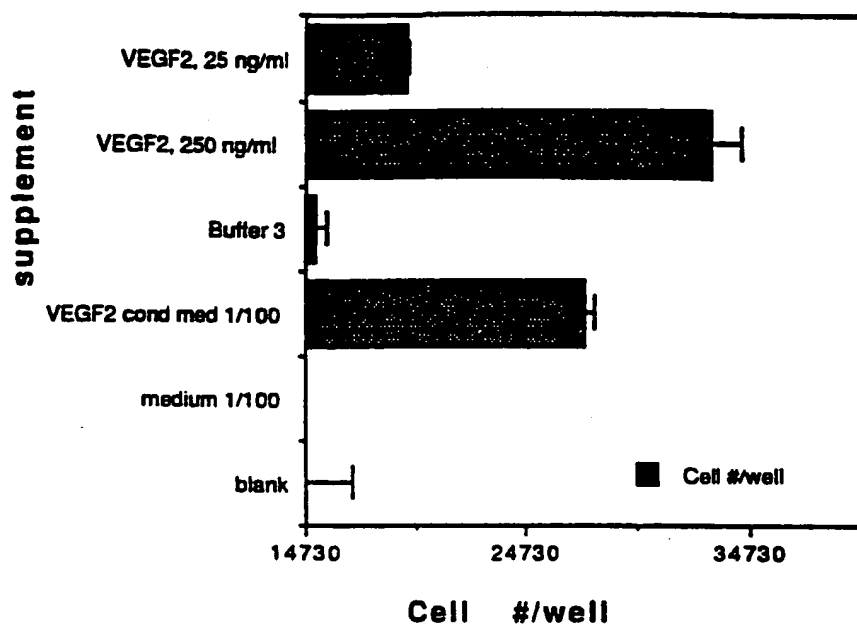


FIGURE 9

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09001

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/12, 15/18, 15/19; C07K 14/475, 14/49, 14/50, 14/65

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 69.4, 172.3, 240.2, 252.3, 320.1; 530/324, 350, 399; 536/23.5, 23.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (files 5, 155, 351, 357, 358) search terms: VEGF1, VEGF2, vascular, endothelial, permeability, factor, growth

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,219,739 (TISCHER ET AL) 15 JUNE 1993, abstract, claims, and figures.	1-2, 6-11
X	US, A, 5,326,695 (ANDERSSON ET AL) 05 JULY 1994, abstract and claims.	1-2, 6-11
A	EP, A, 0,476,983 (MERCK & CO. INC.) 25 MARCH 1992, abstract, claims, and figures.	1-11
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 165, Number 3, issued 29 December 1989, Tischler et al, "Vascular Endothelial Growth Factor: A New Member of the Platelet-Derived Growth Factor Gene Family", pages 1198-1206, see entire document.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search  
20 AUGUST 1996

Date of mailing of the international search report  
05 SEP 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARIANNE PORTA ALLEN

Telephone No. (703) 308-0196



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09001

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-11

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09001

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 69.1, 69.4, 172.3, 240.2, 252.3, 320.1; 530/324, 350, 399; 536/23.5, 23.51

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

- I. Claims 1-11, drawn to nucleic acid sequences for VEGF2, vectors, host cells, methods of production, and the VEGF2 polypeptide, classified in at least Class 536, subclass 23.5, for example.
- II. Claim 12, drawn to VEGF2 agonists, classified in at least Class 530, subclass 399, for example.
- III. Claim 13, drawn to a VEGF2 antagonist, classified in at least Class 530, subclass 399, for example.
- IV. Claim 14, drawn to methods of treatment using the VEGF2 polypeptide, classified in at least Class 514, subclass 2, for example.
- V. Claims 15, drawn to gene therapy methods of treatment, classified in at least Class 514, subclass 44.
- VI. Claim 16, drawn to methods of treatment using the VEGF2 agonist, classified in at least Class 514, subclass 2, for example.
- VII. Claim 17, drawn to methods of treatment using the VEGF2 antagonist, classified in at least Class 514, subclass 2, for example.
- VIII. Claim 18, drawn to methods of diagnosis using the VEGF2 nucleic acid sequences, classified in at least Class 435, subclass 6, for example.
- IX. Claim 19, drawn to methods of diagnosis using the VEGF2 polypeptide, classified in at least Class 435, subclass 7.1, for example.
- X. Claim 20, drawn to methods of identifying compounds, classified in at least Class 435, subclass 7.1, for example.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I forms a single inventive concept including nucleic acid sequences encoding VEGF2 (a first appearing product) and methods of producing the encoded VEGF2 using the nucleic acids (a first appearing method of using the first appearing product). Groups II-III are drawn to structurally different products which do not share the same or a corresponding special technical feature with the first appearing product, the nucleic acid sequences. Groups IV-X are drawn to methods having different goals, method steps, and starting materials which do not share the same or a corresponding special technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application.